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Effect of Gestational Age on the Biochemical Composition of Porcine Placental Glycosaminoglycans[1] (41008)

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Abstract. The effect of increasing gestational age on the biochemical composition of porcine placental glycosaminoglycans (pGAG) was investigated. Glycosaminoglycans (GAG) were isolated from placentas obtained surgically at 20-day intervals during the second half of gestation (Days 50–110) and were characterized by anion-exchange chromatography and cellulose acetate electrophoresis. The dried, defatted porcine placenta contained approximately 60 μg GAG/mg. Isolated pGAG contained heparin, chondroitin sulfates–dermatan sulfate, heparan sulfate, and hyaluronic acid. Hyaluronic acid was the predominant pGAG throughout the second half of gestation and increased from 7.3 μg on Day 50 to 10.4 μg uronic acid/mg dried, defatted placenta on Day 110 ($P < 0.05$) and from 45.6% of total pGAG isolated on Day 50 to 63.9% on Day 110 ($P < 0.05$). Chondroitin sulfates–dermatan sulfate and heparan sulfate decreased ($P < 0.05$) both absolutely and on a percentage basis as gestation progressed. Neither the absolute amount nor the percentage of heparin changed with increasing gestational age.

Glycosaminoglycans (GAG) are anionic, linear carbohydrate polymers composed of specific disaccharide repeating units and are a characteristic component of connective tissue ground substance. The seven GAG found in mammalian tissues are hyaluronic acid (HA), keratan sulfate (KS), heparan sulfate (HS), chondroitin-4-sulfate (C-4-S), chondroitin-6-sulfate (C-6-S), dermatan sulfate (DS), and heparin (HEP). Function(s) of these macromolecules has not been definitely ascertained; however, they appear to be involved in maintenance of connective tissue structural integrity (1, 2), regulation of metabolite transfer through and between tissue compartments (3), and morphogenesis of developing tissues and organs (4–6).

Due to the role of the placenta in nutrient exchange, much of the previous research on placental GAG (pGAG) has focused on the metabolite transfer function of these compounds. This has included the establishment of baseline data on the biochemical composition of pGAG in normal placentas at different stages of gestation. Mouse placentas analyzed throughout the second half of gestation contained HA, HS, C-4-S, C-6-S, DS, HEP, and concentrations of these pGAG varied depending on stage of gestation (7). Human placentas were found to have HA, HS, C-4-S, C-6-S, and DS but no HEP (8). Term human placentas had proportionally more HS and DS than did those collected between 12 and 18 weeks of gestation. It was theorized that these gestational age-related changes might influence nutrient transfer across the placenta.

The purpose of the present investigation was to establish baseline data on the pGAG composition of the porcine epitheliochorial placenta at 20-day intervals during the second half of gestation. These data will serve as the basis for additional biochemical and ultrastructural studies on the functional significance of porcine pGAG.

Materials and methods. Animals and rations. Twenty Hampshire × Yorkshire crossbred gilts were given *ad libitum* feeding of wheat-soybean meal-based rations formulated to meet all known nutrient requirements (9) beginning at 7 weeks of age. A 16% crude protein grower ration was fed from 20 to 95 kg body wt. From 95 kg

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through breeding and gestation, a 14% crude protein ration was fed at the rate of 2 kg per gilt per day. Animals were bred via natural service after completion of at least two normal estrous cycles and placentas were obtained surgically from five animals on each of the following days of gestation (term = 114 days): 50, 70, 90, 110.

Tissue collection and preparation. Following an overnight fast with free access to water, animals were injected with 50 ml saturated KCl solution via the anterior vena cava. Death occurred within 1–2 min at which time the pregnant uterus was removed through a midventral incision. The uterus was oriented as to right and left horns and each horn was incised along the entire length of its respective antimesometrial border. All placentas were removed and trimmed of umbilical vessels at 4°, after which they were weighed and stored at −20°. Forty-eight placentas were obtained from Day 50 gilts, 46 from Day 70 gilts, 44 from Day 90 gilts, and 53 from Day 110 gilts. The average weights of the placentas subsequently used for analyses were 73.3, 102.1, 85.5 and 166.1 g for 50-, 70-, 90-, and 110-day gilts respectively. Thirty grams of each frozen placenta was homogenized in 75% ethanol at 4° using a shearing homogenizer[2] and washed with 75% ethanol at room temperature until centrifugation at 650*g* for 20 min yielded a colorless supernatant. Washed tissue was then defatted with ether–acetone (1/1, v/v) for 72 hr at 4° (10 ml/g frozen placenta removed by centrifugation at 16,300*g* for 20 min every 12 hr). Washed, defatted tissue was dried under vacuum at 60°. All subsequent procedures and analyses were performed on dried, defatted tissue from the middle placentas of each uterine horn (two placentas/animal).

Glycosaminoglycan isolation and characterization. Placental tissue and pGAG from all four stages of gestation studied were processed and analyzed in parallel throughout isolation and characterization procedures. Isolation of pGAG from dried, defatted placentas was performed according to the protocol of Lee and associates (8). Following an initial digestion of tissue with Pronase B at 65° for 24 hr pGAG were precipitated with 5% potassium acetate in 80% ethanol and digested with hog pancreas diastase at 37° for 24 hr to remove any glycogen. Placental GAG recovered from the diastase digestion were further digested with Pronase B for 24 hr at 65° and precipitated with 5% potassium acetate in 80% ethanol. After successive washings with 95% ethanol and diethyl ether, pGAG were dissolved in glass-distilled H_2O, lyophilized, and weighed.

Uronic acid (UA) content of pGAG was determined by the borate–carbazole method of Bitter and Muir (10). Cellulose acetate electrophoresis of pGAG was performed in 0.3 *M* cadmium acetate, pH 4.1, at 4° for 6 hr and membranes were stained with 0.1% Alcian blue G8X in 5% acetic acid–10% ethanol (11). Identification of electrophoresis bands was accomplished by coelectrophoresis of standard GAG and isolated pGAG before and after digestion with testicular hyaluronidase (12) and chondroitinase ABC (12). Quantitation of bands was performed on a Beckman R-112 densitometer and correction for differential dye binding by individual pGAG was made (13). A modification of the cetylpiridinium chloride–cellulose microcolumn technique of Svejcar and Robertson (14) was also used to fractionate pGAG. Seven nominal fractions are eluted by this procedure: F_1 (low-molecular-weight nonsulfate GAG), HA, HS, C-4-S, C-6-S, DS, HEP. This was modified by eluting the C-4-S, C-6-S, and DS fractions as one fraction, CS/DS, using 0.75 *M* $MgCl_2$ in 0.05% cetylpiridinium chloride (14). This allowed microcolumn fractions to correspond to electrophoresis data. Standard GAG were chromatographed in both original (14) and modified systems and were eluted in their nominal fractions. Uronic acid analysis was used to quantitate microcolumn fractions.

Enzymes and standards. Testicular hyaluronidase and chondroitinase ABC were obtained from Sigma Chemical Company, St. Louis, Missouri. Pronase B was purchased from Calbiochem, Los Angeles,

[2] Polytron Kinematica, Brinkman Instruments, Westbury, New York.

California, and hog pancreas diastase was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Standard GAG (DHEW-NIH-NIAMDD Contract No. AM5-2205) were the generous gift of Dr. Martin B. Mathews.

Statistical analyses. Data were analyzed using analysis of variance and Duncan's new multiple range test (15).

Results. pGAG and UA concentrations. Effects of stage of gestation on amount of pGAG isolated, amount of UA per milligram dried, defatted placenta (DDFP) and percentage UA content of pGAG are presented in Table I. Neither the absolute amount of UA in DDFP nor the percentage UA in pGAG was different at the stages of gestation studied. Day 90 placenta was found to have less ($P < 0.05$) pGAG than Day 70 placenta; however, there is no readily apparent explanation for this finding. Since there was no difference in percentage UA content of pGAG at these stages of gestation, it is not likely that the reduced amount of pGAG represents differential extraction.

Electrophoresis. Electrophoresis of a mixture of standard HA, HS, C-4-S, C-6-S, DS, and HEP resulted in five bands which are given in order of increasing mobility: HA, HS, DS, C-4/6-S, HEP. Due to hybridization, it was not always possible to obtain suitable separation of C-4/6-S and DS in isolated pGAG and as a result only four bands have been designated: HA, HS, CS/DS, HEP. In addition, HEP of isolated pGAG was designated as such based on its resistance to chondroitinase ABC even though it migrated slightly ahead of standard HEP. Results of electrophoresis of pGAG are given in Fig. 1. Hyaluronic acid was the major pGAG at all stages of gestation studied and increased, both on a relative and absolute basis, with increasing gestational age. The decrease in absolute amount of HA in Day 90 placenta vs Day 70 placenta (Fig. 1A) can be accounted for by the decreased amount of total pGAG isolated from day 90 placenta (Table I). Both relative and absolute decreases in CS/DS concentration were evident with increasing gestational age. Heparan sulfate decreased with increasing gestational age when expressed on a percentage basis. The amount of HEP did not change as gestation progressed.

Microcolumn fractionation. Fractionation of pGAG in this system did not elute HEP shown to be present by electrophoresis. Since pGAG had slightly greater mobility than standard HEP in the electrophoresis system and therefore a greater negative charge, it was necessary to change the solvent used in the microcolumn fractionation to one of higher ionic strength in order to elute HEP from the columns. Satisfactory recoveries of pGAG HEP were obtained using 6 *N* HCl (16) as the eluting solvent. Results of microcolumn fractionization of pGAG are illustrated in Fig. 2 and they are in close agreement with pGAG electrophoresis results. Hyaluronic acid was again shown to be the predominant pGAG throughout the second half of gestation and increased on a percentage basis with increasing gestational age. Heparan sulfate

TABLE I. EFFECTS OF GESTATIONAL AGE ON AMOUNTS OF ISOLATED PLACENTAL GAG (pGAG) AND URONIC ACID (UA) CONTENT IN DRIED, DEFATTED PLACENTA (DDFP), AND PERCENTAGE UA OF ISOLATED GAG

Stage of gestation	pGAG (% of DDFP)[a]	UA	
		μg/mg DDFP	% of pGAG[b]
Day 50[c]	6.40 ± 0.29^{AB}	16.02 ± 0.78^{A}	25.09 ± 0.81^{A}
Day 70[c]	6.79 ± 0.41^{A}	16.55 ± 1.15^{A}	24.46 ± 0.99^{A}
Day 90[c]	5.18 ± 0.24^{B}	13.91 ± 0.71^{A}	27.17 ± 1.17^{A}
Day 110[c]	6.24 ± 0.33^{AB}	16.18 ± 0.58^{A}	26.31 ± 1.05^{A}

[a] pGAG (% of DDFP) = wt of pGAG (mg)/wt of DDFP (mg) used to isolate pGAG × 100.
[b] UA (% of pGAG) = wt of UA (μg) from standard curve/wt of pGAG analyzed (μg) × 100.
[c] Each value is the mean ± SEM of ten pGAG isolates (2/animal). Means in the same column without a common superscript (AB) are significantly different ($P < 0.05$).

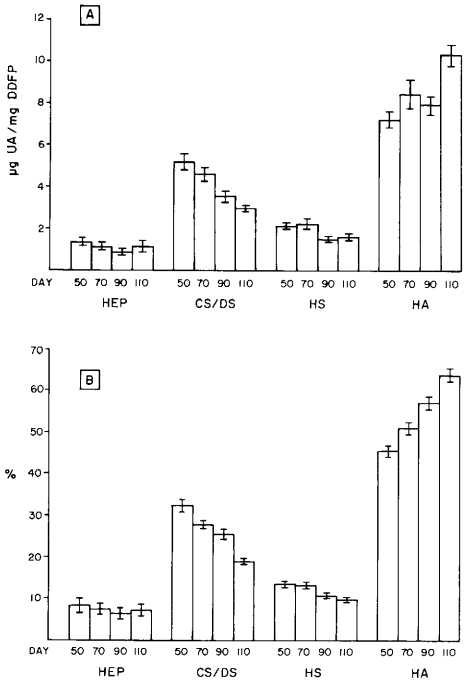

FIG. 1. Effect of gestational age on electrophoresis of pGAG. Values are mean ± SEM of 10 pGAG isolates. Numbers at the bottom of each column are days of gestation. Placental GAG fractions are designated by abbreviations at the bottom of each group of columns: HEP, heparin; CS/DS, chondroitin sulfates/dermatan sulfate; HS, heparan sulfate; HA, hyaluronic acid. (A) Placental GAG fractions expressed as micrograms of UA per milligram of dried, defatted placenta. (B) Placental GAG fractions are expressed as percentage of total pGAG.

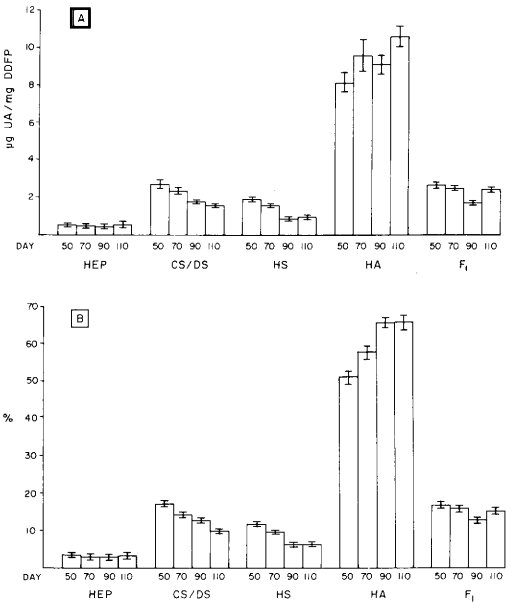

FIG. 2. Effect of gestational age on microcolumn fractionation of pGAG. Values are means ± SEM of 10 pGAG isolates. Numbers at the bottom of each column are days of gestation. Placental GAG fractions are designated by abbreviations at the bottom of each group of columns: HEP, heparin; CS/DS, chondroitin sulfates/dermatan sulfate; HS, heparan sulfate, HA, hyaluronic acid; F_1, low-molecular-weight nonsulfated GAG. (A) Placental GAG fractions expressed as micrograms of UA per milligram of dried, defatted placenta. (B) Placental GAG fractions are expressed as percentage of total pGAG.

and CS/DS both decreased absolutely and relatively as gestation progressed. Heparin concentration did not change with gestational age from 50 to 110 days.

Discussion. The porcine epitheliochorial placenta is an unusually rich source of GAG containing approximately 60 μg GAG/mg DDFP throughout the second half of gestation. This amount is eight times greater than that found in human placenta (8) and two times greater than that in mouse placenta (7). Gestational age-related changes found in the biochemical composition of porcine pGAG differ markedly from those found in mouse (7) and human (8) placenta. Total pGAG content of murine discoid haemochorial placentas, expressed as micrograms of UA/per milligram of DDFP, increased from 3.7 on Day 12 (term = 21 days) to a peak of 9.3 on Day 15 and declined to 1.4 on Day 19 (7). Heparan sulfate was the major pGAG on Day 12 being replaced by HA as the major pGAG on Day 15 and both declined steadily thereafter to Day 19. Dermatan sulfate, C-4-S, C-6-S, and HEP were minor pGAG components of murine placentas and except for a slight transitory increase on Day 16, their concentrations remained fairly constant. Svejcar (7) stated that the large quantities of HA, "must influence the placental permability," but offered no explanation as to how this action might be mediated. More importantly, Svejcar (7) noted that the fractionation pattern of mouse pGAG indicated these compounds were undergoing a significant degree of autolysis at term. Lee

and associates (8) characterized pGAG from young (12–18 weeks gestational age) and term (40 weeks gestational age) human placentas. Young placenta contained more GAG (2.2 μg UA/mg DDFP) than did term placenta (1.55 μg UA/mg DDFP). Unlike porcine pGAG, HA was a minor component of human pGAG and its concentration did not change during gestation. Also in contrast to porcine pGAG, percentages of C-4-S and C-6-S in human pGAG did not change and HS and DS increased with gestational age. The percentage of nonsulfated chondroitin in term human placentas was less than half that in young placentas (9.3 vs 22.5%). Heparin was not detected in human pGAG. Although there is little doubt that the results accurately represent the composition of human pGAG analyzed by Lee *et al.*, it seems appropriate to question these findings, particularly those regarding term pGAG. In light of the evidence of autolysis in term mouse placentas (7) and of extensive pGAG degradation demonstrated ultrastructurally in vaginally delivered as compared to surgically obtained term porcine placentas (V. Steele, unpublished data), it is doubtful that pGAG isolated from vaginally delivered placentas accurately reflects the pGAG content/composition of functioning term placentas.

The functional significance of human pGAG has been considered with regard to its possible influence on nutrient transfer. Calatroni and DiFerrante (17) proposed existence of two GAG barriers in human placental villi: one consisting of large quantities of HS located at the level of the trophoblast and fetal capillaries, the other consisting primarily of DS located in the villous mesenchymal core. Lee and associates (8) theorized that proportional increases of HS and DS found in term vs young human placentas should modify both the chemical and physical nature of ground substance interposed between maternal and fetal circulations thereby altering metabolite transfer through it. However, it is not known to what degree the gestational age-related changes in human pGAG composition reported in that study represent changes occurring in the areas of functional nutrient exchange.

Gestational age-related changes in porcine pGAG composition described in this investigation provide a basis for studies on the functional significance of these compounds. The increase in HA concentration calls attention to itself as the most important pGAG compositional alteration in that it has no precedent in any organ previously studied. Decreasing concentrations of CS/DS and HS with increasing gestational age most probably reflect normal degradation rates of these compounds coupled with their decreased synthesis subsequent to elevated HA synthesis. Hyaluronic acid has been shown to depress glucosamine and sulfate incorporation into CS of cultured embryonic chick chondrocytes, but it has no effect on degradation of preformed CS (18). The significance of pGAG HEP and its lack of response to gestational age are not readily apparent.

Recent studies have noted that synthesis of HA in developing tissues is often associated with occurrence of cell migration (4) and expansion of extracullular spaces (6). It is possible that gestational age-related alterations in porcine pGAG composition may be involved in some aspect of placental development, and therein may lie their functional significance.

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